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MECHANISMS OF RESISTANCE

Differential Penicillin-Binding Protein 5 (PBP5) Levels in the *Enterococcus faecium* Clades with Different Levels of Ampicillin Resistance

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ABSTRACT Ampicillin resistance in *Enterococcus faecium* is a serious concern worldwide, complicating the treatment of *E. faecium* infections. Penicillin-binding protein 5 (PBP5) is considered the main ampicillin resistance determinant in *E. faecium*. The three known *E. faecium* clades showed sequence variations in the *pbp5* gene that are associated with their ampicillin resistance phenotype; however, these changes alone do not explain the array of resistance levels observed among *E. faecium* clinical strains. We aimed to determine if the levels of PBP5 are differentially regulated between the *E. faecium* clades, with the hypothesis that variations in PBP5 levels could help account for the spectrum of ampicillin MICs seen in *E. faecium*. We studied *pbp5* mRNA levels and PBP5 protein levels as well as the genetic environment upstream of *pbp5* in 16 *E. faecium* strains that belong to the different *E. faecium* clades and for which the ampicillin MICs covered a wide range. Our results found that *pbp5* and PBP5 levels are increased in subclade A1 and A2 ampicillin-resistant strains compared to those in clade B and subclade A2 ampicillin-susceptible strains. Furthermore, we found evidence of major clade-associated rearrangements in the region upstream of *pbp5*, including large DNA fragment insertions, deletions, and single nucleotide polymorphisms, that may be associated with the differential regulation of PBP5 levels between the *E. faecium* clades. Overall, these findings highlight the contribution of the clade background to the regulation of PBP5 abundance and point to differences in the region upstream of *pbp5* as likely contributors to the differential expression of ampicillin resistance.

KEYWORDS ampicillin, *Enterococcus faecium*, penicillin-binding protein 5, protein levels, resistance

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Ampicillin-resistant *Enterococcus faecium* has emerged to be one of the leading pathogens in the hospital setting (1, 2). In the United States, the incidence of infections caused by ampicillin-resistant *E. faecium* strains has increased since the 1980s, and this has been accompanied by a progressive increase in the MICs over time (3, 4); currently, the majority of *E. faecium* isolates recovered in hospitals around the United States are resistant to this β-lactam antibiotic (1, 2). The decreased susceptibility of *E. faecium* to ampicillin and other β-lactams has been mainly attributed to the high-molecular-weight penicillin-binding protein 5 (PBP5) (3–5). The proposed mechanism of PBP5-mediated action is a low affinity for β-lactam antibiotics, allowing peptidoglycan synthesis (transpeptidation) and bacterial growth when the other PBPs are inhibited by the drug (6, 7).

High-level ampicillin resistance in clinical isolates of *E. faecium* has been primarily associated with variants of PBP5 with an even lower affinity for β-lactams (8–12). In particular, specific amino acid changes in the C-terminal transpeptidase domain of PBP5 have been associated with a reduced affinity for penicillin and increased β-lactam MICs (8–10). A methionine-to-threonine or methionine-to-alanine substitution at position 485 (Met-485-Thr/Ala) and the addition of aspartic acid or serine after position 466 (Asp-466′ and Ser-466′, respectively), in which both positions are located close to the active site of the enzyme, have been associated with the highest levels of resistance to β-lactam antibiotics (9–13). In addition, other amino acid substitutions, including alanine to isoleucine or alanine to threonine at position 499 (Ala-499-Ile/Thr), glutamine to valine at position 629 (Glu-629-Val), and proline to serine at position 667 (Pro-667-Ser), have been implicated in resistance to β-lactams (9, 10, 12). Rice et al. demonstrated that single substitutions at positions 485, 499, 629, and 466′ had a low impact on the ampicillin MICs, but when these mutations were present in combination, the levels of resistance were amplified (8). However, in clinical isolates, there is not an absolute correlation between these substitutions and high levels of ampicillin resistance (10). In addition, factors other than the PBP5 mutations, including increased PBP5 production, have been suggested to play a role in elevating the MICs of ampicillin and other β-lactams (5, 14–17).

Interestingly, we previously showed that the two main *E. faecium* lineages found in humans, the hospital-associated clade (subclade A1) and the community-associated clade (clade B) (18, 19), differ in their ampicillin susceptibility, which we attributed to the presence of two distinct allelic forms of the *pbp5* gene, whose sequences differ by approximately 5% (10). Most of the subclade A1 strains are resistant to ampicillin (MICs $\geq$ 16 μg/ml) and harbor the consensus *pbp5-R* allele, while we found that the majority of clade B strains are susceptible to ampicillin (MIC $\leq$ 2 μg/ml) and harbor the consensus *pbp5-S* allele (10). In contrast, subclade A2 strains, which are primarily associated with animals and are estimated to have split from subclade A1 75 years ago (18), were shown to display ampicillin MICs that range from 0.5 to 128 μg/ml, with the majority of isolates analyzed harboring a hybrid-like *pbp5* (*pbp5-S/R*) allele, considered an intermediate allele between *pbp5-S* and *pbp5-R* (12). It is interesting to note that, in addition to the amino acid changes in the transpeptidase domain of PBP5 described above, we found that the different *pbp5* alleles have a number of amino acid variations and silent polymorphisms throughout the PBP5 protein and *pbp5* gene, respectively (10, 12).

On the basis of the observation that in some clinical *E. faecium* isolates (presumably from subclade A1) increased amounts of PBP5 have been associated with elevated β-lactams MICs, we postulated that differences in PBP5 levels exist between the *E. faecium* clades; specifically, we inferred that PBP5 abundance would be higher in clade A ampicillin-resistant strains than clade B and subclade A2 ampicillin-susceptible strains. Herein, we examined *pbp5* mRNA and PBP5 protein abundance by Northern and Western blotting and we studied the genetic environment upstream of *pbp5* from 16 *E. faecium* strains from clades A1, A2, and B that carry different variants of the *pbp5* allele and for which the ampicillin MICs covered a wide range.

RESULTS

PBP5 levels are increased in subclade A1 and A2 ampicillin-resistant strains. The 16 *E. faecium* strains and their ampicillin MICs and PBP5 types are shown in Table 1. At times throughout this article, the clade designation of the *E. faecium* strains is written as a subscript after the strain name (for example, the clade B strain Com15 is written $Com15_B$). For the PBP5 type designation, we used the one that we described in the article by Pietta et al. (12) and that is based on the 21 positions previously reported to consistently vary between PBP5-S and PBP5-R strains (S_xR_y, where *x* is the number of positions that match the PBP5-S consensus sequence and *y* is the number of positions that match the PBP5-R consensus sequence; Table 1) (10). Consistent with our previous reports (10, 12), clade B strains showed ampicillin MICs of $\leq$2 μg/ml and subclade A1

TABLE 1 Relevant characteristics of the *E. faecium* strains used in this study

Clade	Strain name	Source (isolation site)	Country/yr of isolation	ST[a]	AMP MIC[b] (μg/ml)	PBP5-S/R type[c]	Reference
B	Com15	Healthy volunteer (feces)	USA/2007	583	0.19	S_{21}/R_0	33
	TX1330	Healthy volunteer (feces)	USA/1994	107	1	S_{20}/R_1	34
	1.141.733	Hospitalized patient (wound)	USA/2005	327	1	S_{20}/R_1	33
	TX2050	Unknown	USA/1971	296	2	S_{20}/R_1	35
	E980	Healthy volunteer (feces)	NLD[d]/1998	94	1	S_{17}/R_4	36
A2	EnGen12	Hospitalized patient (ascites)	NLD/1995	27	0.5	S_{13}/R_8	18
	EnGen35	Hospitalized patient (gut)	NLD/1979	66	1	S_8/R_{13}	18
	EnGen21	Hospitalized patient (feces)	NLD/2002	5	8	S_8/R_{13}	18
	EnGen52	Hospitalized patient (blood)	NLD/2002	332	128	S_8/R_{13}	18
	EnGen24	Hospitalized patient (urine)	NLD/2001	210	32	S_2/R_{19}	18
	EnGen25	Hospitalized patient (stomach)	NLD/1965	92	128	S_0/R_{21}	18
A1	TX16 (DO)	Endocarditis patient (blood)	USA/1992	18	16	S_4/R_{17}	37
	TX82	Endocarditis patient (blood)	USA/1999	17	64	S_1/R_{20}	38
	C68	Hospitalized patient (feces)	USA/1996	16	128	S_1/R_{20}	39
	1.230.933	Hospitalized patient (blood)	USA/2005	18	128	S_1/R_{20}	33
	1.231.502	Hospitalized patient (blood)	USA/2005	203	128	S_1/R_{20}	33

[a]ST, multilocus sequence type.
[b]AMP, ampicillin. Susceptibility testing was performed by broth microdilution, except for strain Com15, where the AMP MIC was determined by Etest.
[c]PBP5-S/R type as described by Pietta et al. (12), where the subscript number after S is the number of positions that match the PBP5-S consensus sequence and the subscript number after R is the number of positions that match the PBP5-R consensus sequence.
[d]NLD, The Netherlands.

strains displayed ampicillin MICs of $\geq$16 μg/ml, while the MICs for the subclade A2 strains ranged from 0.5 to 128 μg/ml (Table 1). It is important to note that although two of the six subclade A2 strains studied had an MIC of 128 μg/ml, they were selected because their MICs were higher than those for the 13 A2 strains, for which the median MIC was 4 μg/ml.

A Western blot analysis with a polyclonal serum raised against recombinant PBP5-S (rPBP5-S) from strain $Com15_B$ showed that PBP5 protein levels were higher in the ampicillin-resistant strains from clade A than the highly ampicillin-susceptible strains from clade B and subclade A2 (Fig. 1). When PBP5 levels were detected with a polyclonal serum against rPBP5-R from strain $C68_{A1}$, comparable results were obtained (Fig. 1 and S1 in the supplemental material), indicating that the differences observed between strains were due to differences in PBP5 protein abundance and not in the affinity of the antibodies for a particular type of PBP5 (Fig. 1 and S1). In contrast, no differences in the RNA polymerase β subunit levels were observed between the strains (Fig. S1). Furthermore, comparable results were obtained in independent experiments (biological and technical replicates; Fig. 1 and S1).

When comparing strains of the same clade or subclade, we observed a correlation between the ampicillin MIC (Table 1), the PBP5 sequence type (Table 1), and the levels of PBP5 protein (Fig. 1 and S1). Within subclade A1, TX16 (strain DO) displays moderate resistance to ampicillin (MIC = 16 μg/ml) (10, 12), which correlates with its reduced PBP5 protein abundance (Fig. 1 and Fig. S1) compared to that of the other subclade A1 strains with higher ampicillin MICs (MICs $\geq$ 64 μg/ml) studied. However, in addition to its lower PBP5 levels than other subclade A1 strains, TX16 (DO) (PBP5-S_4/R_{17}) also lacks two amino acid changes (Asp/Ser-466′ and Met-485-Thr/Ala) that have been linked with the highest levels of ampicillin resistance (10, 12), which could also explain its moderate ampicillin MIC. It is important to note that although we observed higher PBP5 protein levels in all the ampicillin-resistant strains than in the highly susceptible ones (MICs = 0.19 to 1 μg/ml), the correlation between PBP5 protein levels and MICs was not strictly proportional. For example, strain TX82 showed higher PBP5 protein levels than strain 1.230.933, albeit the latter strain has an MIC of 128 μg/ml, while the MIC for the former strain is 64 μg/ml.

Within subclade A2, we analyzed three strains that displayed considerably different ampicillin MICs (EnGen35, EnGen21, and EnGen52, for which the ampicillin MICs were

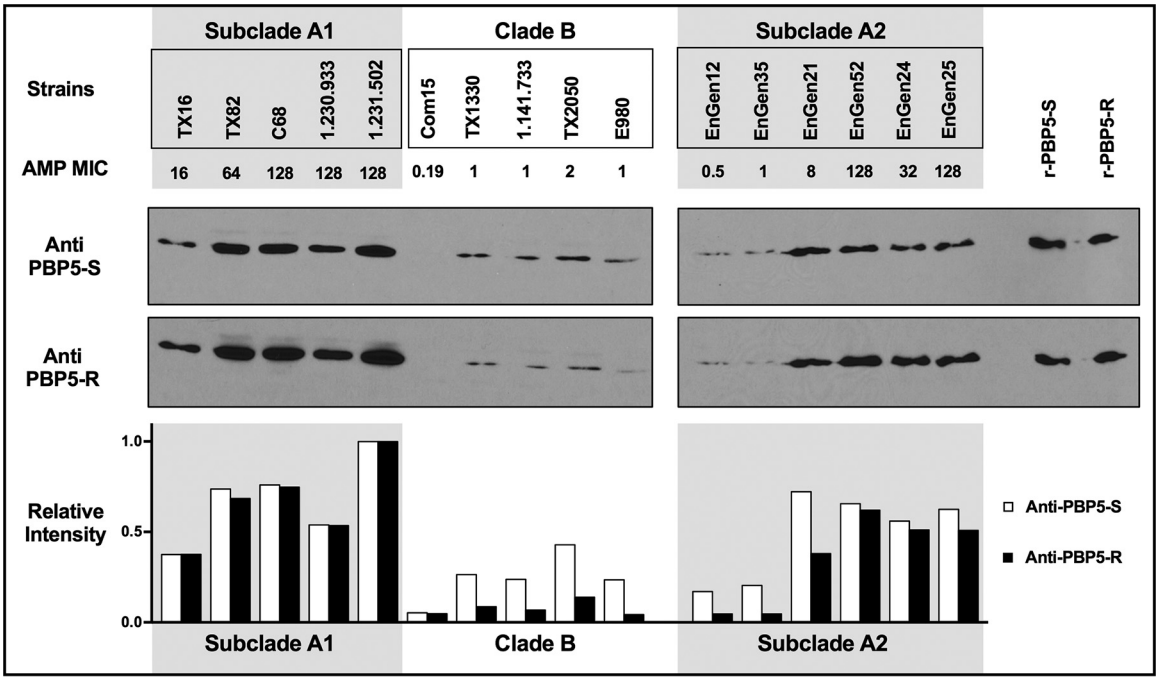

FIG 1 Differential PBP5 levels in the *E. faecium* clades detected by Western blotting. Equivalent protein samples were separated by SDS-PAGE and transferred to a PVDF membrane, and PBP5 was detected with a polyclonal serum raised against rPBP5-S from strain Com15 or rPBP5-R from strain C68 (the antibodies used to detect PBP5-S were removed, and the membrane was reprobed with anti-rPBP5-R). The *E. faecium* strains and ampicillin (AMP) MICs are indicated above the blots (see Table 1 for detailed descriptions of the strains), while the relative quantification of the band intensity for PBP5 normalized to the RNA polymerase β subunit levels is shown below the blots; 10 ng each of recombinant PBP5-S and PBP5-R was loaded into the last two lanes as controls. See Fig. S1 for the results of an independent experiment with the rPBP5-R antiserum and a monoclonal antibody against the RNA polymerase subunit β protein (loading control).

1 μg/ml, 8 μg/ml, and 128 μg/ml, respectively) but that had same number of amino acid changes (PBP-S_8/R_{13}) associated with the PBP5-R form of the protein (EnGen35 and EnGen52 have the same amino acid sequence in these 21 positions, while the amino acid sequence of EnGen21 diverges at one position; see Table S3 in the supplemental material). Interestingly, reduced PBP5 protein levels in EnGen35 may account for its very low MIC compared to the MICs for EnGen21 and EnGen52 (Fig. 1 and S1). Conversely, PBP5 protein levels were comparable between EnGen21 and EnGen52 (Fig. 1 and S1), suggesting that other factors, such as the seven amino acid differences (six of which are outside the 21 consensus positions) between the PBP5 sequences of these two strains (12), could also play a role in the different ampicillin MICs (Table S4). The PBP5 sequence type may also account for the observed differences in MICs between EnGen24 (MIC = 32 μg/ml) and EnGen25 (MIC = 128 μg/ml), as both strains showed similar PBP5 protein levels (Fig. 1 and S1). When clade B strains were compared to each other, similar low PBP5 protein levels were observed in protein preparations from strains TX1330, 1.141.733, TX2050, and E980 (MICs = 1 to 2 μg/ml; Fig. 1 and S1). On the other hand, Com15 showed a reduction in the PBP5 quantity (Fig. 1 and S1; the band was visible only when the film was overexposed), which is in accordance with its hypersusceptibility (MIC = 0.19 μg/ml; Table 1).

We also studied *pbp5* mRNA levels by Northern hybridization with a 218-bp probe designed to anneal to a region of *pbp5-S* and *pbp5-R* that is identical in the two allelic forms of the gene except at 1 nucleotide. In accordance with the Western blotting results, the *pbp5* mRNA levels were higher in all the ampicillin-resistant strains from subclades A1 and A2 than the ampicillin-susceptible strains from clade B and subclade A2 (Fig. S2). For certain strains, however, we did not observe complete agreement between the PBP5 protein level (Fig. 1 and Fig. S1) and the *pbp5* mRNA level (Fig. S2). For example, EnGen35 showed a higher relative *pbp5* mRNA level than EnGen12; however, both strains exhibited a similar PBP5 protein abundance. The absence of a

TABLE 2 Summary of major changes observed upstream of *pbp5* in the 16 *E. faecium* strains used in this study

Clade	Strain name	AMP[a] MIC (μg/ml)	Size of *psr* gene (bp)	Size of *psr-pbp5* intergenic region (bp)	Insertion upstream of *pbp5*	Genetic environment pattern	GenBank accession no.
B	Com15	0.19	885	130	No	A	NZ_GG670325
	TX1330	1	885	130	No	A	NZ_GG669057
	1.141.733	1	885	130	No	A	NZ_GG688461
	TX2050	2	885	130	No	A	
	E980	1	885	130	No	A	NZ_ABQA01000020
A2	EnGen12	0.5	885	125	No	A[b]	NZ_KB029389
	EnGen35	1	885	125	No	A[b]	NZ_KB029791
	EnGen21	8	885[c]	1,471	Yes (1 gene)[d]	B	NZ_KB029603
	EnGen52	128	756[e]	57[e]	No	C	NZ_KB029984
	EnGen24	32	756	57	No	C	NZ_KB029871
	EnGen25	128	756	57	No	C	NZ_KB029748
A1	TX16 (DO)	16	885	1,471	Yes (1 gene)[d]	B	NC_017960
	TX82	64	798[f]	2,555[f]	Yes (3 genes)	D	NZ_GL455979
	C68	128	757[g]	57	No	E	NZ_LPUE01000001
	1.230.933	128	798[f]	2,555[f]	Yes (3 genes)	D	NZ_GG692537
	1.231.502	128	756	57	No	C	NZ_GG688486

[a]AMP, ampicillin.
[b]Deletion of 5 nucleotides in the intergenic region of *psr* and *pbp5* compared to the sequence of clade B strains.
[c]Annotated as a pseudogene.
[d]A 1,179-bp transposase inserted within the intergenic region of *psr* and *pbp5*.
[e]Our PCR and sequencing results do not match the sequence reported in the NCBI database for strain EnGen52 (accession number NZ_KB029984).
[f]The exact size in these strains was not determined, but the presence of the 3 genes inserted between *psr* and *pbp5* was confirmed by PCR and sequencing using the primers listed in Table S2 in the supplemental material; the size reported is based on the sequence for strain Aus0004 (GenBank accession number NC_017022) available in the NCBI database.
[g]An insertion of 1 nucleotide within the *psr* coding sequence generates a frameshift that results in an early stop codon.

strict correlation between mRNA and protein levels is not unexpected, since posttranscriptional, translational, and posttranslational regulatory networks could govern protein abundance (20). In fact, it has been demonstrated that certain genes with comparable mRNA levels showed up to a 20-fold difference in abundance at the protein level (21).

Rearrangements in the upstream region of *pbp5* may be associated with the differential PBP5 levels. The *pbp5* gene has been demonstrated to exist as part of an operon along with two genes located upstream, *ftsW* and *psr*, with suggested roles in cell wall synthesis (22). We first analyzed *in silico* the sequence upstream of *pbp5* in 15 of the 16 *E. faecium* strains included in this study for which whole-genome sequences are available in the NCBI database (no whole-genome sequence is available for TX2050), followed by confirmation by PCR and Sanger sequencing, using the primers listed in Table S2. When we amplified the region upstream of *pbp5* from the 16 *E. faecium* strains included in this study, using primers *ftsW*3p-F and *pbp5*-R-$^{115-134}$, which anneal on *ftsW* and *pbp5*, respectively, we found that the highly susceptible strains from clade B and subclade A2 (MICs = 0.19 to 2 μg/ml) yield a fragment of approximately 1,280 bp, as detected by PCR (Fig. S3). In contrast, using the strains with reduced ampicillin susceptibility (MICs $\geq$ 8 μg/ml), fragments with a size notably different from that of the fragment of approximately 1,280 bp that correlated with an increased PBP5 abundance were amplified (Fig. S3). The different genetic environments found in the 16 *E. faecium* strains included in this study are summarized in Table 2 and Fig. 2.

One of the changes associated with the highest ampicillin MICs was a 201-bp deletion (confirmed by PCR and sequencing) in five strains, including four of the five strains tested with an ampicillin MIC of 128 μg/ml (EnGen52$_{A2}$, EnGen25$_{A2}$, C68$_{A1}$, and 1.231.502$_{A1}$; Fig. S3) and one strain (Engen24$_{A2}$) with an ampicillin MIC of 32 μg/ml. This deletion encompasses 137 bp of the 3′ end of *psr* and 64 bp of the intergenic region of *psr* and *pbp5* (Table 2; Fig. 2C and E and S4). As this deletion also includes the *psr* stop codon (TAA) and the next stop codon in frame (TAG) is within 8 nucleotides (Fig. S4), the size of the *psr* gene, as shown in Table 2 and Fig. 2, in strains EnGen52$_{A2}$, EnGen24$_{A2}$, EnGen25$_{A2}$, and 1.231.502$_{A1}$ is 756 bp (885 bp − 137 bp + 8 bp = 756 bp).

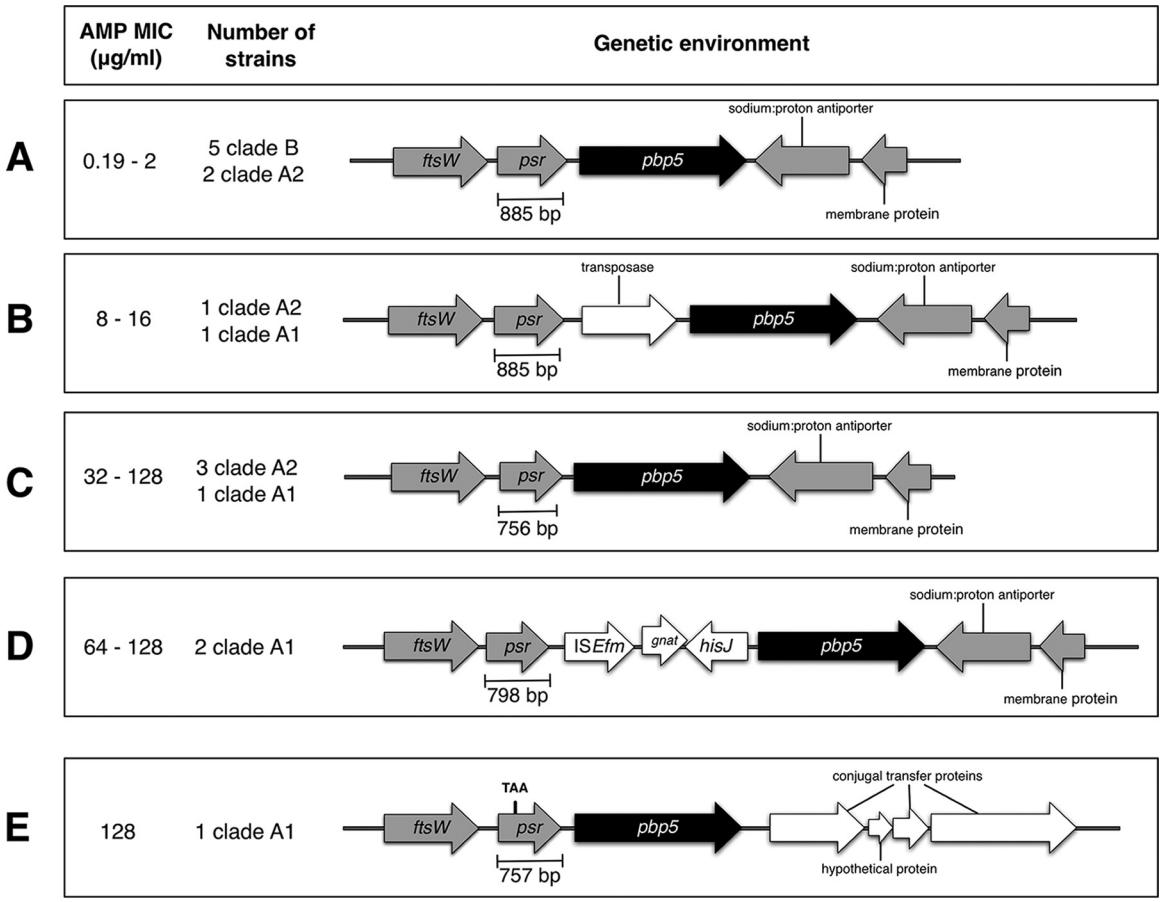

FIG 2 *pbp5* genetic environments found in the 16 *E. faecium* strains included in this study. (A) $Com15_B$, $TX1330_B$, $1.141.733_B$, $TX2050_B$, $E980_B$, $EnGen12_{A2}$, and $EnGen35_B$; (B) $EnGen21_{A2}$ and $TX16\ (DO)_{A1}$; (C) $EnGen52_{A2}$, $EnGen24_{A2}$, $EnGen25_{A2}$, and $1.231.502_{A1}$; (D) $TX82_{A1}$ and $1.230.933_{A1}$; (E) $C68_{A1}$.

Strain $C68_{A1}$ has, in addition to the missing 201 bp, an insertion of 1 nucleotide in the *psr* coding sequence, resulting in an early stop codon (23) (Table 2 and Fig. 2E). In the other two highly resistant strains, $TX82_{A1}$ and $1.230.933_{A1}$ (MICs = 64 to 128 μg/ml, respectively), a >4,000-bp PCR product was prominently obtained (Fig. S3). When we analyzed the region upstream of *pbp5* in the draft genomes of $TX82_{A1}$ and $1.230.933_{A1}$, we found that the sequences in this area contain a series of gaps; however, alignments designed to close these gaps (the primers used are listed in Table S2), along with PCR and sequencing results, indicated a genetic environment similar to that in the subclade A1 strain Aus0004 (Table 2 and Fig. 2D), which is also highly resistant (MIC = 256 μg/ml, as described previously [24] and confirmed here). The region between *psr* and *pbp5* in Aus0004 includes three different open reading frames (ORFs), IS*Efm1*, *gnat*, and *hisJ*, predicted to encode a transposase, an acetyltransferase, and a histidinol phosphate phosphatase, respectively (Fig. 2D). In $TX16_{A1}$ and $EnGen21_{A2}$ (MICs = 16 and 8 μg/ml, respectively), a different insertional event, namely, a 1,346-bp fragment that includes a 1,179-bp transposase with 99% identity between these two strains was found upstream of *pbp5* (Table 2; Fig. 2B and S3). In addition, several other small insertions, deletions, and single nucleotide polymorphisms between clades that could potentially affect PBP5 expression were found (data not shown). For example, a single nucleotide deletion between the demonstrated *pbp5* transcriptional start (22) and the predicted ATG translational start site was observed in clade A ampicillin-resistant strains when their sequences were compared with those of the clade B and subclade A2 ampicillin-susceptible strains. In addition, a 5-bp deletion in the intergenic region of *psr* and *pbp5* was observed in the clade A background compared to the sequence of the clade B

background (Table 2). In contrast, all the strains from clade B had a full-length *psr* gene located immediately upstream of *pbp5*, and no deletions or insertions in the *pbp5-psr* intergenic region were observed (Table 2 and Fig. 2A).

DISCUSSION

High-level ampicillin resistance in *E. faecium* has been primarily associated with sequence mutations in the transpeptidase domain of the low-affinity PBP5 (8–12). It has been demonstrated that some of these substitutions decrease the affinity of the protein for penicillin and correlate with elevated MICs, in particular, when they are present in combination (8). However, *pbp5* sequence differences alone cannot explain the array of different ampicillin MICs seen in *E. faecium* (10, 12), which indicates that other factors can influence ampicillin resistance. In fact, Zhang et al. identified additional genes that contribute to elevate ampicillin MICs (17). Earlier reports also pointed to overproduction of PBP5 as a contributor to resistance, in particular, in isolates with intermediate MICs (16 to 64 μg/ml) (5, 13, 14).

The results presented in this study indicate that, in addition to the different *pbp5* allelic types (different *pbp5* sequences) carried by the *E. faecium* clades (10, 12), there are marked differences in PBP5 abundance that, in the majority of the strains, correlate with their ampicillin resistance phenotype. We found that ampicillin-resistant strains, including highly resistant strains from subclades A1 and A2 with MICs of $>$64 μg/ml, showed higher *pbp5* and PBP5 expression levels than ampicillin-susceptible (MICs = 0.19 to 1 μg/ml) clade B and subclade A2 strains (Fig. 1, S1, and S2). This is in contrast to what was reported by Rybkine et al., who found an apparent decrease in PBP5 protein levels in isolates with MICs of greater than 16 μg/ml (9). Conversely, our results are in accordance with previous findings by Fontana et al., who reported that PBP5 protein levels are elevated in highly ampicillin-resistant strains (14). The controversial role of PBP5 overproduction in ampicillin resistance might be explained, at least in part, by the fact that most of the earlier studies used radiolabeled penicillin as a measure of PBP5 quantity, an experimental approach that also measures the ability of PBP5 to bind to the labeled compound (14), which has been shown to be decreased by the presence of specific PBP5 mutations (8).

The region upstream of *pbp5* has been suggested to contribute to ampicillin resistance. Initial evidence for a role of this region came from studies in *Enterococcus hirae*, where an 87-bp deletion in the 5′ region of the open reading frame (ORF) located upstream of *pbp5* was associated with increased PBP5 levels in a resistant mutant. This finding led to the designation of this ORF as the penicillin-binding protein synthesis repressor (*psr*) (25); however, a direct role of Psr in *pbp5* repression has not yet been established (22). Interestingly, Massidda et al. showed that Psr is involved in the regulation of different cell surface-related processes in *E. hirae*, including lysozyme sensitivity, autolysis, and the levels of rhamnose in the cell wall (26). Furthermore, a role for the region upstream of *pbp5* in the *E. faecium* subclade A1 strain C68 was also shown (22). The ampicillin MICs were higher when the *pbp5* gene of $C68_{A1}$ was cloned along with its upstream region containing *ftsW* and *psr* than when the gene was cloned in the same vector with only its own promoter region (MICs, 64 to 128 μg/ml versus 8 to 16 μg/ml) (22). However, these authors suggest that in strain $C68_{A1}$, Psr does not seem to participate in the regulation of *pbp5* transcription (22). The role of *ftsW* is unknown, but studies of a homologous gene in *Escherichia coli* showed that the product of this gene is a transmembrane protein that interacts with PBP3 in *E. coli* (27). Here, we presented evidence of extensive differences in the region upstream of the *pbp5* gene among the strains studied that may account for the differential abundance of PBP5, including large and small DNA fragment insertions and deletions. Of note, although these deletions and insertional events likely affect the promoter region of *pbp5*, specifically, the region upstream of the putative −10 sequence predicted by Rice et al. (22), one cannot discard the possibility of a role of full-length Psr as a repressor of *pbp5* expression, since all the strains with ampicillin MICs of $\geq$8 μg/ml had altera-

tions in the sequence of the *psr* gene (Table 2) that could render this protein nonfunctional.

Overall, the results presented in this study provide further insight into PBP5-mediated resistance, highlighting that, in addition to amino acid sequence alterations in PBP5, elevated levels of this protein are also observed in highly resistant strains from clade A. In addition, this finding underscores the contribution of the clade background in the regulation of PBP5 abundance and points to differences in the upstream region of *pbp5* as likely contributors to the differential levels of PBP5. Further studies are warranted in order to elucidate the underlying mechanism for the differential regulation of PBP5 between the *E. faecium* clades.

MATERIALS AND METHODS

Bacterial strains, plasmids, routine growth conditions, and susceptibility testing. The relevant characteristics of the *E. faecium* and *Escherichia coli* strains used in this study are described in Table 1 and S1 in the supplemental material, respectively. The *E. faecium* strains were routinely grown at 37°C in brain heart infusion (BHI; Becton, Dickinson [BD], Franklin Lakes, NJ) broth or agar. *E. coli* strains were cultured at 37°C using Luria-Bertani (LB) broth or agar (BD). Ampicillin susceptibility testing was performed by broth microdilution in cation-adjusted Mueller-Hinton II broth (BD) following Clinical and Laboratory Standards Institute (CLSI) guidelines or by Etest (bioMérieux, Marcy-l'Etoile, France). Kanamycin (50 μg/ml; Sigma-Aldrich, St. Louis, MO) was used for the selection of *E. coli* with the pET28a(+) vector and derivatives (Table S1).

Expression and purification of soluble recombinant PBP5-S from Com15 and PBP5-R from C68. Fragments of *pbp5-S* and *pbp5-R* were amplified from the genomic DNA of *E. faecium* strains Com15 and C68, respectively, using primers F-rPBP5 and R-rPBP5, containing the NdeI and BamHI restriction sites, respectively (Table S2). These fragments, lacking the first 108 bp of the *pbp5* coding sequence that corresponds to the 36 amino acids encoding the transmembrane domain, were cloned into the pET28a(+) vector using the NdeI and BamHI sites (Table S1). The resulting recombinant plasmids, pTEX4302.1 (carrying *pbp5-S* from Com15) and pTEX2193.1 (carrying *pbp5-R* from C68), were propagated in *E. coli* TG1 cells and then transformed into *E. coli* BL21(DE3) cells for expression (Table S1). *E. coli* BL21(DE3) strains harboring plasmids pTEX4302.1 and pTEX2193.1 were grown at 37°C in LB medium containing 50 μg/ml kanamycin to mid-log phase. Expression was induced at 16°C by the addition of β-D-1-thiogalactopyranoside to 0.4 mM and an additional incubation for 17 h. A frozen cell pellet was suspended in buffer A [50 mM Tris, pH 7.4, 0.5 M NaCl, 20 mM imidazole, 2 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 5% (vol/vol) glycerol] and an EDTA-free cOmplete protease inhibitor cocktail tablet (Roche Diagnostics Corp., Indianapolis, IN). After sonication (Branson sonifier 250; VWR Scientific, Houston, TX) and centrifugation, the supernatant was loaded onto a HiTrap affinity (Ni^{2+}) column (GE Healthcare Life Sciences, Pittsburgh, PA). The column was washed with 10 column volumes of buffer A and eluted with a continuous elution gradient from 20 to 1 M imidazole. The fractions containing the protein of interest were pooled and dialyzed overnight at 4°C against 50 mM Tris, pH 7.5, 300 mM NaCl, 0.2 mM PMSF, 2 mM TCEP, 5% (vol/vol) glycerol. The N-terminal 6×His tag was removed by treatment with His-tagged tobacco etch virus protease. PBP5 without the 6×His tag was purified from the reaction mixture using the same chromatography strategy described above. The fractions containing the protein of interest were pooled and dialyzed against 50 mM Tris, pH 7.5, 0.2 mM EDTA, 2 mM TCEP, 5% (vol/vol) glycerol, and the sample was further purified through a Q-XL Sepharose column (GE Healthcare Life Sciences) using a 0.1 to 1 M NaCl gradient. The peak fractions were pooled, dialyzed against 50 mM Tris, pH 7.4, 200 mM NaCl, 0.2 mM EDTA, 2 mM TCEP, 5% (vol/vol) glycerol, concentrated, and loaded onto a Superdex-200 preparation-grade column (HiLoad 16/60; GE Healthcare Life Sciences) for the final purification step. Peak fractions were pooled, and the sample purity was assessed by SDS-PAGE and found to be >95%.

Generation of anti-rPBP5-S and anti-rPBP5-R antibodies. Polyclonal antibodies against rPBP5-S and rPBP5-R were separately generated using a previously described scheme (28, 29) and following a protocol and guidelines preapproved by the Animal Welfare Committee of The University of Texas Health Science Center, Houston, TX. In brief, male Sprague-Dawley rats were subcutaneously injected at multiple sites with 1 mg of rPBP5-S or rPBP5-R suspended in Freund's complete adjuvant (FCA) at day 1, followed by two booster doses of 1 mg of rPBP5-S or rPBP5-R prepared in Freund's incomplete adjuvant (FIA) at days 14 and 28. Blood was collected at day 42, followed by euthanasia of the animals. Antibody titers were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (29). Prior to their use, the anti-rPBP5-S and anti-rPBP5-R antibodies were absorbed with *E. coli* BL21(DE3) lysates to remove any anti-*E. coli* antibodies, if present.

PBP5 detection by Western blotting. Cells grown overnight in BHI broth were inoculated into fresh BHI broth at a starting optical density at 600 nm (OD_{600}) of 0.05 and grown at 37°C with gentle shaking until they reached an OD_{600} of 1.0 ± 0.1. The cultures were centrifuged at 3,900 rpm for 10 min, and the pellets were rapidly chilled on dry ice and stored at −80°C until they were used. Cells were resuspended in 1 ml of 1× phosphate-buffered saline and disrupted twice by the use of 1-min bead-beating pulses at maximum speed, followed by centrifugation to recover the supernatant. Protein concentrations were measured using a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. Samples that were normalized so that the protein concentrations were equal were boiled in SDS-containing sample buffer and separated by SDS-PAGE (10%

acrylamide). Coomassie staining was used to ensure that equivalent amounts of protein were loaded into each lane (data not shown). Samples were transferred to a polyvinylidene difluoride (PVDF) membrane (EMD Millipore, Darmstadt, Germany) and analyzed with rat antiserum absorbed against PBP5-S and PBP5-R, followed by incubation with peroxidase-conjugated goat anti-rat IgG, light chain specific (Jackson ImmunoResearch Laboratories, West Grove, PA). As positive controls, 10 ng each of the purified recombinant PBP5-S and PBP5-R proteins was loaded into a lane. As a loading control, a monoclonal antibody targeting RNA polymerase subunit β was used (Santa Cruz Biotechnology, Heidelberg, Germany), followed by detection with peroxidase-conjugated $F(ab')_2$ fragment of donkey anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA). The intensity of the bands was analyzed using ImageJ software (http://imagej.nih.gov/ij/).

RNA extraction and Northern blot analysis. Cultures grown to an OD_{600} of 1.0 $\pm$ 0.1, as described above for the PBP5 Western blot assay, were used for RNA extraction, performed as previously described (30). Primers F-*pbp5*-218 and R-*pbp5*-218 (Table S2) were used for generating a *pbp5* hybridization probe, which amplifies a fragment that is identical in 217 of the 218 nucleotides between *pbp5-S* and *pbp5-R*. Northern blot analyses were carried out as reported before (31, 32).

Analyses of the genetic environment upstream of the *pbp5* gene. Whole-genome sequences for 15 of the 16 strains included in this study were available on the NCBI website (http://www.ncbi.nlm.nih.gov/genome) and were used to retrieve the upstream region of *pbp5*. DNA and protein multiple-sequence alignments were performed using the alignment tool MUSCLE from the EBI website (http://www.ebi.ac.uk/Tools/msa/muscle/). The results of the bioinformatic analyses were confirmed by PCR and Sanger sequencing using the primers listed in Table S2.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02034-16.

TEXT S1, PDF file, 1.9 MB.

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